



# Regulation of $\text{Na}^+/\text{Mg}^{2+}$ antiport in rat erythrocytes

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## Abstract

In rat erythrocytes, the regulation of  $\text{Na}^+/\text{Mg}^{2+}$  antiport by protein kinases (PKs), protein phosphatases (PPs), intracellular  $\text{Mg}^{2+}$ , ATP and  $\text{Cl}^-$  was investigated. In untreated erythrocytes,  $\text{Na}^+/\text{Mg}^{2+}$  antiport was slightly inhibited by the PK inhibitor staurosporine, slightly stimulated by the PP inhibitor calyculin A and strongly stimulated by vanadate. PMA stimulated  $\text{Na}^+/\text{Mg}^{2+}$  antiport. This effect was completely inhibited by staurosporine and partially inhibited by the PKC inhibitors Ro-31-8425 and BIM I. Participation of other PKs such as PKA, the MAPK cascade, PTK, CK I, CK II, CAM II-K, PI 3-K, and MLCK was excluded by use of inhibitors.  $\text{Na}^+/\text{Mg}^{2+}$  antiport in rat erythrocytes can thus be stimulated by  $\text{PKC}\alpha$ .

In non- $\text{Mg}^{2+}$ -loaded erythrocytes, ATP depletion reduced  $\text{Mg}^{2+}$  efflux and PMA stimulation in NaCl medium. A drastic activation of  $\text{Na}^+/\text{Mg}^{2+}$  antiport was induced by  $\text{Mg}^{2+}$  loading which was not further stimulated by PMA. Staurosporine, Ro-31-8425, BIM I and calyculin A did not inhibit  $\text{Na}^+/\text{Mg}^{2+}$  antiport of  $\text{Mg}^{2+}$ -loaded cells. Obviously, at high  $[\text{Mg}^{2+}]_i$   $\text{Na}^+/\text{Mg}^{2+}$  antiport is maximally stimulated.  $\text{PKC}\alpha$  or PPs are not involved in stimulation by intracellular  $\text{Mg}^{2+}$ . ATP depletion of  $\text{Mg}^{2+}$ -loaded erythrocytes reduced  $\text{Mg}^{2+}$  efflux and the affinity of  $\text{Mg}^{2+}$  binding sites of the  $\text{Na}^+/\text{Mg}^{2+}$  antiporter to  $\text{Mg}^{2+}$ . In non- $\text{Mg}^{2+}$ -loaded erythrocytes  $\text{Na}^+/\text{Mg}^{2+}$  antiport essentially depends on  $\text{Cl}^-$ .  $\text{Mg}^{2+}$ -loaded erythrocytes were less sensitive to the activation of  $\text{Na}^+/\text{Mg}^{2+}$  antiport by  $[\text{Cl}^-]_i$ .

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**Keywords:**  $\text{Na}^+/\text{Mg}^{2+}$  antiport; Rat erythrocyte;  $\text{PKC}\alpha$ ;  $[\text{Mg}^{2+}]_i$ ; ATP;  $[\text{Cl}^-]_i$

## 1. Introduction

$\text{Na}^+/\text{Mg}^{2+}$  antiport can be regulated by intracellular  $\text{Mg}^{2+}$ , ATP and  $\text{Cl}^-$ . Evidence is as follows.

**Abbreviations:** BIM I, bisindolylmaleimide I; CAM II-K, calmodulin-dependent kinase II; CK I, casein kinase I; CK II, casein kinase II; DAG, diacylglycerol; 2,3 BPG, 2,3 bisphosphoglycerate; GAPDH, glyceraldehyde-phosphate dehydrogenase; MAPK, mitogen-activated protein kinase; MLCK, myosine light chain kinase; PI 3-K, phosphatidylinositol-3 kinase; PK, protein kinase; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; PKG, cGMP-dependent protein kinase; PLC, phospholipase C; PMA, phorbol-12-myristate-13-acetate; PP, protein phosphatase; PP 1, protein phosphatase 1; PP 2A, protein phosphatase 2A; PTP 1B, protein tyrosine phosphatase 1B; PTK, protein tyrosine kinase; Sp-5,6-DCI-cBIMPS, 5,6 dichloro-1- $\beta$ -D-ribofuranosylbenzimidazole 3',5'-cyclic monophosphorothiorate, sp-isomer, sodium salt; TCA, trichloroacetic acid; TRPM, transient receptor potential melastatin-like channel subfamily;  $[\text{X}]_i$ , intracellular concentration of a cation or an anion

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$\text{Na}^+/\text{Mg}$  antiport is drastically activated by loading erythrocytes with  $\text{Mg}^{2+}$  [1,2]. In analogy to  $\text{Na}^+/\text{Mg}^{2+}$  antiport,  $\text{Na}^+/\text{H}^+$  antiport is activated by intracellular  $\text{H}^+$  [3–5], and  $\text{Na}^+/\text{Ca}^{2+}$  antiport by intracellular  $\text{Ca}^{2+}$  [6–8].

$\text{Na}^+/\text{Mg}^{2+}$  antiport is dependent on ATP. Inhibition of energy metabolism reduced the activity of  $\text{Na}^+/\text{Mg}^{2+}$  antiport in chicken erythrocytes [9]. In rat [10] and human [11,12] erythrocytes, ATP depletion reduced  $\text{Na}^+/\text{Mg}^{2+}$  antiport and reversed  $\text{Na}^+/\text{Mg}^{2+}$  antiport in rat erythrocytes [13] by decreasing the affinity of the  $\text{Na}^+/\text{Mg}^{2+}$  antiporter for intracellular  $\text{Mg}^{2+}$ . This was shown by an increase in log  $K$  in the Hill plot [13]. Like  $\text{Na}^+/\text{Mg}^{2+}$  antiport, other secondary active transport systems, e.g.  $\text{Na}^+/\text{H}^+$  antiport,  $\text{Na}^+/\text{Ca}^{2+}$  antiport,  $\text{Na}^+/\text{K}^+/\text{Cl}^-$  symport and  $\text{K}^+/\text{Cl}^-$  symport, also depend on ATP [14–17]. The mechanism of action of ATP in these transport systems has been controversially discussed [14–16]. The  $\text{Na}^+/\text{K}^+/\text{Cl}^-$  symporter is active when two threonines are phosphorylated [17]. Recently, it was found that the  $\text{Na}^+/\text{Ca}^{2+}$  antiporter is phosphorylated by PKA and PKC in the form of a macromolecular complex [16].

The effect of ATP in  $\text{Na}^+/\text{Mg}^{2+}$  antiport may be mediated by phosphorylation of the  $\text{Na}^+/\text{Mg}^{2+}$  antiporter similarly as in  $\text{Na}^+/\text{Ca}^{2+}$  antiport [16] and  $\text{Na}^+/\text{K}^+/\text{Cl}^-$  symport [17]. Amiloride-sensitive phosphorylation of an erythrocyte membrane protein has already been found [18]. In line with these results, it was possible to stimulate  $\text{Na}^+/\text{Mg}^{2+}$  antiport in various cell types by effectors that stimulate phosphorylation, for example in rat erythrocytes by a phorbol ester [13], and in thymocytes [19], ascites cells [20] and liver cells [21] by a cell permeant cAMP analog, which activates PKA. In rat liver cells,  $\text{Mg}^{2+}$  efflux by  $\text{Na}^+/\text{Mg}^{2+}$  antiport evoked following stimulation of  $\beta$ -adrenergic receptors [21,22]. The same was observed when hepatocytes were stimulated by forskolin [21]. In  $\text{Mg}^{2+}$ -deprived cultured mouse distal convoluted tubule cells (MDCT), isoproterenol [23], Br-cAMP [24],  $\text{PGE}_2$  [25] and adenosine [26] stimulated  $\text{Mg}^{2+}$  influx via  $\text{Na}^+/\text{Mg}^{2+}$  antiport. Also, in perfused rat heart,  $\text{Na}^+/\text{Mg}^{2+}$  antiport was stimulated by  $\beta$ -adrenergics [27,28]. These results indicate a role of PKA in the activation of  $\text{Na}^+/\text{Mg}^{2+}$  antiport of several tissues.

Stimulation of  $\alpha_1$ -adrenergic receptors induced  $\text{Mg}^{2+}$  efflux from rat hepatocytes [29], a  $\text{Na}^+$ -dependent one via  $\text{Na}^+/\text{Mg}^{2+}$  antiport and a  $\text{Ca}^{2+}$ -dependent one [30]. This was taken as evidence for a PLC signaling pathway resulting in activation of PKC by the increased production of DAG. Thus, in addition to PKA,  $\text{Na}^+/\text{Mg}^{2+}$  transport can also be affected by PKC.

Finally, it should be noted that in  $\text{Mg}^{2+}$ -loaded renal epithelial cells,  $\text{Na}^+/\text{Mg}^{2+}$  antiport was activated by nitric oxide and cGMP [31]. This points to a possible third way of regulating  $\text{Na}^+/\text{Mg}^{2+}$  antiport via PKG.

We previously found stimulation of  $\text{Na}^+/\text{Mg}^{2+}$  antiport by intracellular  $\text{Cl}^-$  in non- $\text{Mg}^{2+}$ -loaded rat erythrocytes. In analogy,  $\text{Na}^+/\text{H}^+$  antiport and  $\text{Na}^+/\text{K}^+/\text{Cl}^-$  symport were stimulated by intracellular  $\text{Cl}^-$  (for literature, see Ref. [32]). It has been proposed that the major effect of  $[\text{Cl}^-]_i$  on  $\text{Na}^+/\text{K}^+/\text{Cl}^-$  symport is exerted via a volume-sensitive protein kinase (PK) [33].

Similar to other transport processes, these findings show that there is a regulation of  $\text{Na}^+/\text{Mg}^{2+}$  antiport by various PKs and by the intracellular concentrations of  $\text{Mg}^{2+}$ , ATP and  $\text{Cl}^-$ . The different activation mechanisms may be interrelated as shown for the interaction of intracellular  $\text{Mg}^{2+}$  and cAMP in the activation of  $\text{Na}^+/\text{Mg}^{2+}$  antiport from thymocytes [19].

We therefore investigated the interplay of intracellular  $\text{Mg}^{2+}$ , ATP and  $\text{Cl}^-$  with various PKs, PPs in the regulation of  $\text{Na}^+/\text{Mg}^{2+}$  antiport of rat erythrocytes. In order to exclude a possible ineffectivity in rat erythrocytes, several inhibitors were used at various concentrations for each PK or PP. As shown in previous studies by us, rat erythrocytes were suitable for studying  $\text{Mg}^{2+}$  efflux without  $\text{Mg}^{2+}$  loading [1]. It was therefore possible to directly compare  $\text{Mg}^{2+}$  efflux of non- $\text{Mg}^{2+}$ -loaded and  $\text{Mg}^{2+}$ -loaded cells and the effect of  $\text{Mg}^{2+}$  loading.

## 2. Materials and methods

### 2.1. Materials

Nembutal, sodium solution was obtained from Abott Labs., N. Chicago, IL, USA. A 23187 from Molecular Probes, Eugene, Or., USA, isoproterenol, and sodium orthovanadate from Sigma, Taufkirchen, Germany. The inhibitors or stimulators of the various PKs and PPs were from Calbiochem Novabiochem GmbH, Bad Soden, Germany. All other chemicals were purchased from Merck Eurolab GmbH, Darmstadt, Germany at the highest grade of purity available. Filtered, deionized and virtually  $\text{Mg}^{2+}$ -free water with a resistance of 15–18  $\text{M}\Omega/\text{cm}$  was used for solutions.

### 2.2. Red cell preparation and incubation

By catheterizing the abdominal vein with a heparinized syringe, blood (6–8 ml) was obtained from one anesthetized male Sprague Dawley rat (50 mg/kg Nembutal® i.p.), weighing 350–450 g. The blood was transferred to several heparinized tubes and diluted 1:5 with a solution containing (in  $\text{mmol l}^{-1}$ ) 150 NaCl, 5 glucose, 10 HEPES–Tris, pH 7.4 (NaCl medium). The cell suspension was centrifuged at  $1000 \times g$  for 10 min. The supernatant plasma and buffy coat containing the white cells were aspirated and discarded. The red cells were washed twice in 10-ml NaCl medium. Finally, the red cells were suspended as a 10% suspension (v/v) in NaCl, NaSCN, KCl, or KSCN medium containing (in  $\text{mmol l}^{-1}$ ) 150 of each salt, 5 glucose, 10 HEPES–Tris, pH 7.4. Paired experiments were conducted throughout in the different media with various inhibitors or stimulators.

To avoid hemolysis, the cells were handled with great caution. The experimental temperature was therefore set to 24 °C, and low-speed centrifugation was used.

### 2.3. ATP depletion

ATP depletion of rat erythrocytes was performed by substituting 5  $\text{mmol l}^{-1}$  D-glucose in the media during washing and incubation by 5  $\text{mmol l}^{-1}$  deoxyglucose. Furthermore, 1  $\text{mmol l}^{-1}$  iodoacetate was added to the media.

### 2.4. $\text{Mg}^{2+}$ loading

The cells were loaded with  $\text{Mg}^{2+}$  by incubating a 10% (v/v) cell suspension for 30 min at 37 °C in  $\text{Mg}^{2+}$  loading medium containing (in  $\text{mmol l}^{-1}$ ) 140 KCl, 50 sucrose, 5 D-glucose and 10 HEPES–Tris, pH 7.4, 12  $\text{MgCl}_2$  and 6  $\mu\text{mol l}^{-1}$  A 23187 (dissolved in dimethyl sulfoxide). The slightly hypertonic loading medium protected the cells against hemolysis. After loading, the ionophore was removed by incubating the cells four times in ionophore-free  $\text{Mg}^{2+}$  loading medium plus 1% bovine serum albumin for 10

min at 37 °C. Thereafter, the erythrocytes were washed two times in the desired cooled isotonic incubation media.

### 2.5. $Mg^{2+}$ efflux

$Mg^{2+}$  transport was determined by measuring the increase of  $Mg^{2+}$  concentration in the incubation medium. At the beginning and end of an experiment, 1-ml aliquots of the cell suspensions were centrifuged at  $1000 \times g$  for 10 min. Unless otherwise indicated, the incubation at 24 °C lasted 2 h in non- $Mg^{2+}$ -loaded erythrocytes and 10 to 30 min in  $Mg^{2+}$ -loaded erythrocytes. To determine  $Mg^{2+}$ , the supernatant was diluted with 5% (w/v) TCA, containing 0.1% (w/v)  $La_2O_3$  and 0.16% (v/v) HCl.  $Mg^{2+}$  was measured in triplicate by means of atomic absorption spectrometry (Perkin Elmer, 2380).  $Mg^{2+}$  efflux was calculated from the increase in extracellular  $Mg^{2+}$  concentration during the time interval, and was related to the original cell volume measured with hematocrit. Hematocrit and hemolysis were measured in each sample.  $Mg^{2+}$  efflux was corrected for hemolysis. For this purpose, TCA was added to the sedimented erythrocytes and the  $Mg^{2+}$  content of the TCA extract was determined. Where hemolysis was greater than 2%, the red cells were not taken for the experiment.

### 2.6. Statistical analysis

Data were expressed as mean values  $\pm$  S.E., and statistical differences were determined using Student's paired and two-tailed *t* test. A value of  $P < 0.05$  was considered significant.

## 3. Results and discussion

### 3.1. $Mg^{2+}$ efflux from untreated erythrocytes

Initially, the effect of PKA, PKC, other PKs and PPs on  $Mg^{2+}$  efflux was investigated in non- $Mg^{2+}$ -loaded erythrocytes. PKA is present in human [34,35] and rabbit erythrocytes [36,37]. To test whether PKA is involved in the regulation of  $Na^+/Mg^{2+}$  antiport, Sp-5,6-DCI-cBIMPS ( $0.5$ – $10 \mu\text{mol l}^{-1}$ ), acting as a phosphodiesterase stable activator, and H 89 ( $100$ – $1000 \text{ nmol l}^{-1}$ ), acting as an inhibitor, were tried. Both drugs had no effect on  $Mg^{2+}$  efflux. Furthermore, the ACCase stimulators isoproterenol ( $10$ – $100 \mu\text{mol l}^{-1}$ ) and forskolin ( $100$ – $250 \mu\text{mol l}^{-1}$ ), which by increased generation of cAMP could stimulate PKA, also had no significant effect on  $Mg^{2+}$  efflux (data not shown). An explanation for the failing effect of isoproterenol and forskolin is that, in contrast to human erythrocytes [34,35], rat erythrocytes do not contain  $\beta$ -adrenergic receptors, which are lost during maturation [38]. From these results we can exclude a role of PKA in  $Na^+/Mg^{2+}$  antiport of rat erythrocytes.

Besides PKA,  $PKC_\alpha$  and phorbol-ester-insensitive PKC isoforms are present in human erythrocytes [39–41]. Unfortunately, in rat erythrocytes the PKC isoforms have not been characterized. To determine whether  $Mg^{2+}$  efflux from non-PMA-treated rat erythrocytes is regulated by PKC, we tested the unspecific PK inhibitor staurosporine and BIM I, a more specific inhibitor of the nPKC and cPKC group. As can be seen from Table 1, staurosporine ( $1 \mu\text{mol l}^{-1}$ ) inhibited  $Mg^{2+}$  efflux in NaCl medium by about 15% and by 20% in NaSCN medium. BIM I ( $1 \mu\text{mol l}^{-1}$ ) had no significant effect in NaCl but slightly stimulated  $Mg^{2+}$  efflux by 14% in NaSCN. The reason for the stimulation of  $Mg^{2+}$  efflux in NaSCN medium by BIM I is not clear. The  $PKC_\alpha$  inhibitor resveratrol ( $1$ – $25 \mu\text{mol l}^{-1}$ ) and Gö 6976 ( $250$ – $1000 \text{ nmol l}^{-1}$ ) had no effect on  $Mg^{2+}$  efflux (data not shown). These results indicate that  $PKC_\alpha$  is not involved in  $Mg^{2+}$  efflux in non-PMA-treated rat erythrocytes. Therefore, in untreated erythrocytes,  $Mg^{2+}$  efflux should be modulated by PKs other than PKA and  $PKC_\alpha$ .

To our knowledge, there exists no report of any presence of PKG in rat erythrocytes, which could also be involved in the regulation of  $Na^+/Mg^{2+}$  antiport. A role of PKG was excluded by using PK inhibitors, which were also reported as inhibiting PKG. These inhibitors were emodin, genistein, H89, lavendustin A, and PP2. All these substances were without effect on  $Mg^{2+}$  efflux (data not shown).

In erythrocytes, the presence of various PTKs such as CK I [42] and CK II [43],  $PTK_{p72syk}$  [44] and  $PTK_{p56/53lyn}$  [45], PI 3-K [41], MLCK [46], and p38MAPK [47] has been verified. Staurosporine sensitivity has been documented for some of them. Several of the PKs are involved in the regulation of electrolyte transport. In human erythrocytes the phosphorylation state of the anion exchanging band 3 protein at tyrosine residues is determined by the combined action of the PTKs p72syk and p56/53lyn in opposite to PTP 1B [48]. Also, CK I phosphorylated band 3 protein at threonine residues [42], while CK II phosphorylated several structural proteins as spectrin and others, but not band 3 protein [43]. In human erythrocytes the NHE1 isoform of

Table 1

Effect of the PK inhibitors staurosporine and BIM I and of the PP inhibitors okadaic acid and calyculin A on  $Mg^{2+}$  efflux of non-PMA-treated rat erythrocytes in NaCl medium or NaSCN medium

Group	NaCl medium ( $\mu\text{mol l cells}^{-1}$ 2 h $^{-1}$ )	<i>P</i>	NaSCN medium ( $\mu\text{mol l cells}^{-1}$ 2 h $^{-1}$ )	<i>P</i>
Control	251.3 $\pm$ 7.2		83.7 $\pm$ 5.2	
Staurosporine, 1.0 $\mu\text{mol l}^{-1}$	214.6 $\pm$ 2.8	<0.0001	67.3 $\pm$ 1.1	0.0006
BIM I, 1.0 $\mu\text{mol l}^{-1}$	259.1 $\pm$ 4.7	n.s.	95.2 $\pm$ 4.7	0.0008
Okadaic acid, 0.5 $\mu\text{mol l}^{-1}$	259.9 $\pm$ 7.7	0.037	91.4 $\pm$ 4.8	0.0096
Calyculin A, 0.5 $\mu\text{mol l}^{-1}$	272.7 $\pm$ 19.1	0.011	98.9 $\pm$ 6.2	0.0002

Means $\pm$ S.E. of five paired experiments.

the  $\text{Na}^+/\text{H}^+$  antiporter is activated by PI 3-K, which phosphorylates PKC $\zeta$  [41]. A role of MLCK or a volume-sensitive kinase in the regulation of  $\text{Na}^+,\text{K}^+,\text{Cl}^-$  co-transport has been discussed [33,46]. MAPK is involved in the regulation of NHE1 [47].

To differentiate which of these PKs might control  $\text{Na}^+/\text{Mg}^{2+}$  antiport, various inhibitors were tested in non-PMA-treated erythrocytes. These were IC261 ( $1\text{--}100\text{ }\mu\text{mol l}^{-1}$ ) for CK I, Ly 29402 ( $1\text{--}50\text{ }\mu\text{mol l}^{-1}$ ) and emodin ( $10\text{--}50\text{ }\mu\text{mol l}^{-1}$ ) for CK II, tyrphostin B46 ( $1\text{--}10\text{ }\mu\text{mol l}^{-1}$ ), herbimycin A ( $0.1\text{--}10\text{ }\mu\text{mol l}^{-1}$ ), PP2 ( $10\text{ nmol l}^{-1}\text{--}10\text{ }\mu\text{mol l}^{-1}$ ), lavendustin A ( $25\text{ nmol l}^{-1}\text{--}10\text{ }\mu\text{mol l}^{-1}$ ) for PTK $_{\text{src}}$ , PTK $_{\text{p72 syk}}$  and PTK $_{\text{p56/53 lyn}}$ , KN-62 ( $0.1\text{--}10\text{ }\mu\text{mol l}^{-1}$ ) for CAM II-K, Ly 294102 ( $1\text{--}50\text{ }\mu\text{mol l}^{-1}$ ) for PI 3-K, ML-7 ( $100\text{--}1000\text{ nmol l}^{-1}$ ) for MLCK, SB 203580 ( $20\text{--}1000\text{ nmol l}^{-1}$ ), U0126 ( $20\text{--}1000\text{ nmol l}^{-1}$ ), PD 98059 ( $100\text{--}1000\text{ nmol l}^{-1}$ ) for the MAPK cascade. These inhibitors had no effect on  $\text{Mg}^{2+}$  efflux in non-PMA-treated erythrocytes (data not shown). Thus, in non-PMA-stimulated erythrocytes, the staurosporine-sensitive PK activity that affects  $\text{Mg}^{2+}$  efflux could not be identified.

To investigate the role of PPs in  $\text{Mg}^{2+}$  efflux of untreated erythrocytes, we tested whether the inhibition of protein phosphatases (PPs) present in erythrocytes such as the isoenzyme PP1 $\alpha$  [49], PP 2A [50], and PTP 1B [48] would increase  $\text{Mg}^{2+}$  efflux. PTP 1B is associated with band 3 protein and can be activated by increasing  $[\text{Mg}^{2+}]_i$  [48]. All these PPs are involved in the regulation of  $\text{K}^+,\text{Cl}^-$  symport [48–50]. As shown in Table 1 (see above), okadaic acid acting as an inhibitor of erythrocyte PP 2A only slightly increased  $\text{Mg}^{2+}$  efflux in NaCl medium by 3% and by 9% in NaSCN medium. Calyculin A, an inhibitor of both PP1 and PP 2A, showed a more pronounced inhibition of  $\text{Mg}^{2+}$  efflux, which was 9% in NaCl medium and 18% in NaSCN medium. This may be taken to indicate that in non-PMA-treated rat erythrocytes the serine/threonine phosphatase PP1 participates in the regulation of  $\text{Na}^+/\text{Mg}^{2+}$  antiport, while the role of PP 2A is less clear. In human erythrocytes, calyculin A was also a stronger inhibitor of  $\text{K}^+,\text{Cl}^-$  symport than okadaic acid [50]. The smaller effect of okadaic acid compared to calyculin A may be caused by the experimental temperature used. At  $25\text{ }^\circ\text{C}$ , calyculin A was a more potent inhibitor because it permeates more rapidly than okadaic acid [51].

The contribution of PTP 1B to  $\text{Mg}^{2+}$  efflux in non-PMA-treated rat erythrocytes was investigated by using vanadate. It has been reported that in human erythrocytes vanadate inhibited PTP 1B followed by a strong increase in band 3 phosphorylation, [44,48,52]. As shown in Fig. 1, in NaCl medium orthovanadate drastically increased  $\text{Mg}^{2+}$  efflux five times more than PMA, as depicted in Fig. 3 (see below). The  $K_m$  of orthovanadate amounted to  $55\text{ }\mu\text{mol l}^{-1}$  (not shown) and was in the concentration range that inhibited PTP [48,52]. Unfortunately, orthovanadate is a very unspecific effector. It has digitalis-like effects on heart muscle and induces insulin-like effects [53,54]. Further-

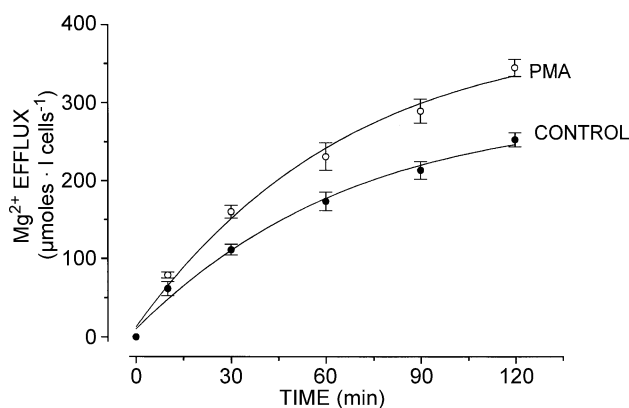


Fig. 1. Increase of  $\text{Mg}^{2+}$  efflux ( $\Delta\text{Mg}^{2+}$  efflux) by increasing concentrations of orthovanadate in non- $\text{Mg}^{2+}$ -loaded rat erythrocytes suspended in NaCl medium.  $\Delta\text{Mg}^{2+}$  efflux was calculated as the difference of  $\text{Mg}^{2+}$  efflux at the particular orthovanadate concentration minus  $\text{Mg}^{2+}$  efflux of the control without vanadate. Means  $\pm$  S.E.,  $n = 3\text{--}5$ .

more, vanadate inhibits various enzymes such as PTP, PP 1, PP 2A, alkaline and acid phosphatases [55–57], glycolytic enzymes, e.g. GAPDH [58], and activates adenylate cyclase [54]. In erythrocytes, vanadate is rapidly taken up and inhibits glycolysis resulting in a reduction of the concentrations of ATP and 2,3 BPG, which are the major  $\text{Mg}^{2+}$  chelators in erythrocytes. Due to the reduction of ATP and 2,3 BPG, bound  $\text{Mg}^{2+}$  will thus be liberated, which increases  $[\text{Mg}^{2+}]_i$ . The increase in  $[\text{Mg}^{2+}]_i$  in vanadate-treated erythrocytes was shown directly by  $^{31}\text{P}$ -NMR [59]. Since increased  $[\text{Mg}^{2+}]_i$  activates  $\text{Na}^+/\text{Mg}^{2+}$  antiport (Ref [1], and see below), the stimulation of  $\text{Mg}^{2+}$  efflux by vanadate may be caused to a minor extent by the inhibition of PPs, but mainly by the increase of  $[\text{Mg}^{2+}]_i$ .

### 3.2. Stimulation of $\text{Mg}^{2+}$ efflux by PMA in non- $\text{Mg}^{2+}$ -loaded erythrocytes

Treatment of erythrocytes with  $1\text{ }\mu\text{mol l}^{-1}$  PMA stimulated  $\text{Mg}^{2+}$  efflux. Fig. 2 shows the time course of PMA stimulation of  $\text{Mg}^{2+}$  efflux. It can be seen that the PMA effect was stable throughout the experimental period of 2 h. Fig. 3A shows the increase of  $\text{Mg}^{2+}$  efflux in NaCl medium and NaSCN medium ( $\Delta\text{Mg}^{2+}$  efflux) at different PMA concentrations. Plotting the values of Fig. 3A according to Lineweaver–Burk yielded  $K_m$  values for PMA of 51 and  $53\text{ nmol l}^{-1}$ , and  $V_{\text{max}}$  values of  $83.3$  and  $43.7\text{ }\mu\text{mol l cells}^{-1}\text{ h}^{-1}$  in NaCl medium and NaSCN medium, respectively (Fig. 3B). The identical  $K_m$  values indicate that PMA is bound to the membrane with the same affinity in NaCl and NaSCN medium. PMA increased  $\text{Mg}^{2+}$  efflux in NaCl medium by 42%, and in NaSCN by 47% (mean values from Figs. 3A, 7 and 8). The relative stimulation of  $\text{Mg}^{2+}$  efflux by PMA was almost the same in both media. However, the absolute value of stimulation in NaCl medium was twice that in NaSCN medium. This may be due to the different lyotropic properties of  $\text{Cl}^-$  and  $\text{SCN}^-$  (see Section 3.6).



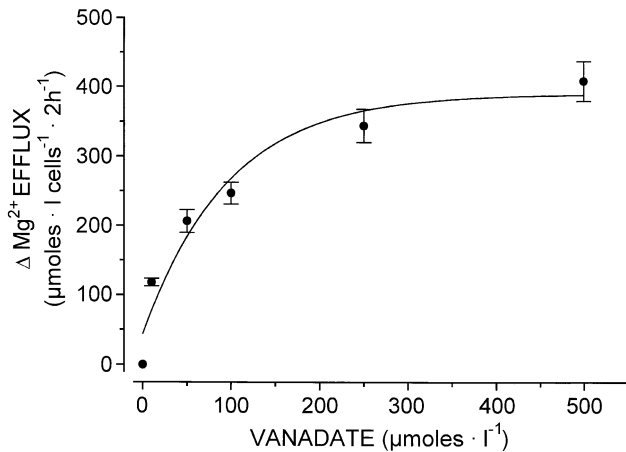


Fig. 2. Time dependence of  $\text{Mg}^{2+}$  efflux of non- $\text{Mg}^{2+}$ -loaded rat erythrocytes suspended in NaCl medium in controls or cells treated with  $1 \mu\text{mol l}^{-1}$  PMA. Means  $\pm$  S.E.,  $n=3-4$ .

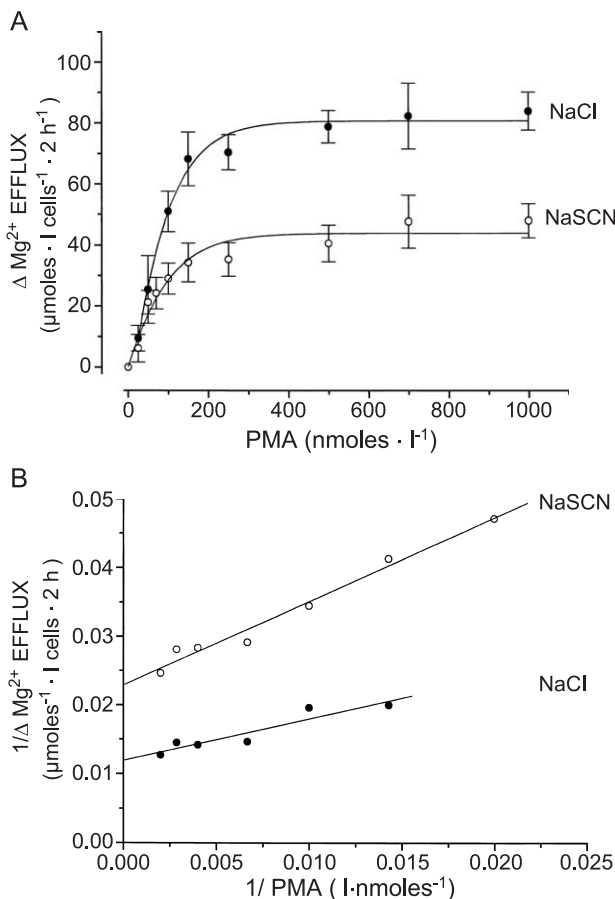


Fig. 3. (A) Increase of  $\text{Mg}^{2+}$  efflux ( $\Delta\text{Mg}^{2+}$  efflux) by increasing concentrations of PMA in non- $\text{Mg}^{2+}$ -loaded rat erythrocytes suspended in NaCl medium or NaSCN medium.  $\text{Mg}^{2+}$  efflux in untreated controls amounted to  $217.6 \pm 4.5 \mu\text{mol l cells}^{-1} 2 \text{ h}^{-1}$  in NaCl medium and  $90.3 \pm 3.4 \mu\text{mol l cells}^{-1} 2 \text{ h}^{-1}$  in NaSCN medium. Means  $\pm$  S.E.,  $n=4-9$ . (B) Lineweaver-Burk plot of PMA-dependent increase of  $\text{Mg}^{2+}$  efflux ( $\Delta\text{Mg}^{2+}$  efflux) in non- $\text{Mg}^{2+}$ -loaded rat erythrocytes in NaCl medium or NaSCN medium. Data were taken from (A). The  $K_m$  value for PMA was  $51 \text{ nmol l}^{-1}$  in NaCl and  $53 \text{ nmol l}^{-1}$  in NaSCN medium. Means  $\pm$  S.E.,  $n=4-9$ .

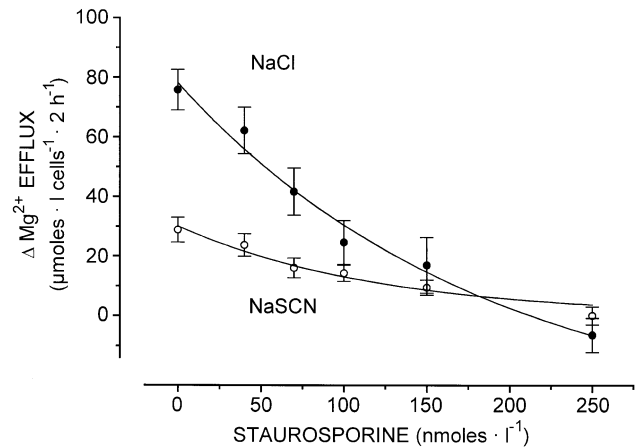


Fig. 4. Inhibition of PMA-stimulated  $\text{Mg}^{2+}$  efflux ( $\Delta\text{Mg}^{2+}$  efflux) of non- $\text{Mg}^{2+}$ -loaded rat erythrocytes in NaCl and NaSCN medium by staurosporine.  $\Delta\text{Mg}^{2+}$  efflux was calculated as the difference of  $\text{Mg}^{2+}$  efflux at the particular concentration of staurosporine plus PMA minus  $\text{Mg}^{2+}$  efflux of the controls without PMA and without staurosporine. Means  $\pm$  S.E.,  $n=5-9$ .

It was possible to completely inhibit the PMA-induced stimulation of  $\text{Mg}^{2+}$  efflux by staurosporine in NaCl and NaSCN medium (Fig. 4). Staurosporine decreased the  $\text{Mg}^{2+}$  efflux below that of non-PMA-treated erythrocytes. This agrees with the effect of staurosporine in Table 1. The  $\text{IC}_{50}$  values of staurosporine in NaCl medium and NaSCN medium amounted to  $196$  and  $199 \text{ nmol l}^{-1}$ , respectively (not shown). The PKC inhibitor Ro-31-8425 dose-dependently inhibited that part of the  $\text{Mg}^{2+}$  efflux that was induced by PMA ( $\Delta\text{Mg}^{2+}$  efflux) (Fig. 5). The  $\text{IC}_{50}$  value amounted to  $46.5 \text{ nmol l}^{-1}$  in NaCl and  $50 \text{ nmol l}^{-1}$  in NaSCN medium (not shown). Furthermore, with increasing concentrations of the specific PKC inhibitor BIM I, PMA stimulation was inhibited, but not completely abolished

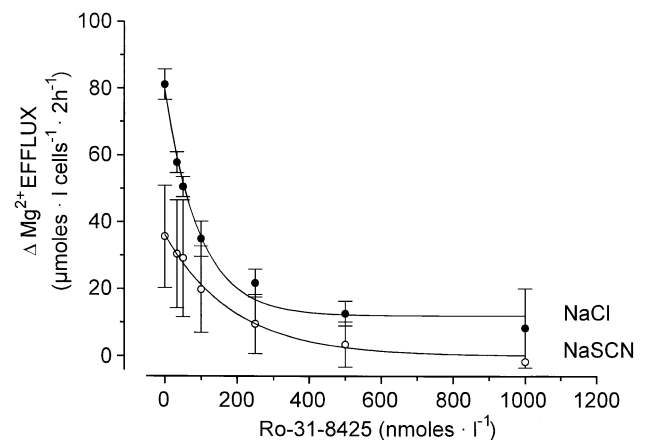


Fig. 5. Inhibition of PMA-stimulated  $\text{Mg}^{2+}$  efflux ( $\Delta\text{Mg}^{2+}$  efflux) of non- $\text{Mg}^{2+}$ -loaded rat erythrocytes suspended in NaCl or NaSCN medium by Ro-31-8425.  $\Delta\text{Mg}^{2+}$  efflux was calculated as the difference of  $\text{Mg}^{2+}$  efflux at the particular concentration of Ro-31-8425 plus PMA minus  $\text{Mg}^{2+}$  efflux of the controls without PMA and without Ro-31-8425. Means  $\pm$  S.E.,  $n=4-6$ .

(Fig. 6). Similarly, the  $\text{Na}^+/\text{H}^+$  antiporter was stimulated by  $1 \mu\text{mol l}^{-1}$  PMA by 35% and staurosporine was a more effective inhibitor of  $\text{Na}^+/\text{H}^+$  antiport than the more specific PKC inhibitor Ro-31-8220 [60]. Small differences between BIM I, Ro-31-8425 and Ro-31-8220 may be due to small differences in the structure and specificity of these bisindolylmaleimide inhibitors.

Besides representing a membrane receptor for PKC, PMA has additional effects. PMA can serve as a non-kinase phorbol ester/DAG receptor for  $\alpha_1$ ,  $\alpha_2$ ,  $\beta_1$ ,  $\beta_2$  chimaerine and Ras-GRP [61]. These are proteins with a cysteine-rich phorbol ester/DAG binding domain. They do not possess PK activity but have a C-terminal GTPase activating domain, which accelerates GTP hydrolysis through small GTP binding proteins. In the activation of MAPK, phorbol esters mediate the binding of RAS-GAP to the plasma membrane [61]. Therefore, it was of interest to test the effect of MAPK and PTK inhibitors on PMA stimulation. A further reason is that the human erythrocyte MAPK is involved in insulin activation of the NHE1 isoform of the  $\text{Na}^+/\text{H}^+$  antiporter [47]. The MAPK cascade inhibitors SB 203580 ( $20\text{--}1000 \text{ nmol l}^{-1}$ ), U0126 ( $20\text{--}1000 \text{ nmol l}^{-1}$ ), PD 89059 ( $100\text{--}1000 \text{ nmol l}^{-1}$ ) did not inhibit the stimulation of  $\text{Mg}^{2+}$  efflux by PMA. Also the PTK inhibitors tyrphostin B46 ( $1\text{--}10 \mu\text{mol l}^{-1}$ ), herbimycin A ( $0.1\text{--}10 \mu\text{mol l}^{-1}$ ), PP2 ( $10 \text{ nmol l}^{-1}\text{--}10 \mu\text{mol l}^{-1}$ ), lavendustin A ( $25 \text{ nmol l}^{-1}\text{--}10 \mu\text{mol l}^{-1}$ ) for PTK<sub>src</sub>, PTK<sub>p72 syk</sub> and PTK<sub>p56/53 lyn</sub> had no effect (data not shown). It can thus be excluded that the MAPK cascade and PTK play a role in PMA-stimulated  $\text{Mg}^{2+}$  efflux via  $\text{Na}^+/\text{Mg}^{2+}$  antiport.

Since staurosporine is a broad-spectrum inhibitor of several PKs, we tested the effect of other PK inhibitors on the stimulation of  $\text{Mg}^{2+}$  efflux by PMA. These were: IC261 ( $1\text{--}100 \mu\text{mol l}^{-1}$ ) for CK I, Ly 29402 ( $1\text{--}50 \mu\text{mol l}^{-1}$ ) and emodin ( $10\text{--}50 \mu\text{mol l}^{-1}$ ) for CK II, KN-62 ( $0.1\text{--}10 \mu\text{mol l}^{-1}$ ) for CAM II-K, Ly 294102 ( $1\text{--}50 \mu\text{mol l}^{-1}$ ) for PI 3-K, and ML-7 ( $100\text{--}1000 \text{ nmol l}^{-1}$ ) for MLCK. These inhibitors did not inhibit PMA-stimulated  $\text{Mg}^{2+}$  efflux (data not shown). Consequently, a role of all these PKs in regulating  $\text{Na}^+/\text{Mg}^{2+}$  antiport is excluded.

Table 2

Effect of okadaic acid and calyculin A on  $\text{Mg}^{2+}$  efflux of PMA-stimulated rat erythrocytes suspended in NaCl medium or NaSCN medium

Group	NaCl medium ( $\mu\text{mol l cells}^{-1}$ $2 \text{ h}^{-1}$ )	<i>P</i>	NaSCN medium ( $\mu\text{mol l cells}^{-1}$ $2 \text{ h}^{-1}$ )	<i>P</i>
Control	251.3±15.2		105.0±24.1	
PMA, $1 \mu\text{mol l}^{-1}$	347.2±25.4	0.0114 <sup>a</sup>	158.0±30.3	0.0058 <sup>a</sup>
PMA, $1 \mu\text{mol l}^{-1}$ + okadaic acid, $0.5 \mu\text{mol l}^{-1}$	358.9±26.2	0.0304 <sup>b</sup>	178.0±35.3	0.0175 <sup>b</sup>
PMA, $1 \mu\text{mol l}^{-1}$ + calyculin A, $0.5 \mu\text{mol l}^{-1}$	321.9±38.9	0.0049 <sup>b</sup>	169.1±14.6	n.s. <sup>b</sup>

Means±S.E. of six paired experiments.

<sup>a</sup> Compared to control.

<sup>b</sup> Compared to PMA.

$\text{l}^{-1}$ ) for CAM II-K, Ly 294102 ( $1\text{--}50 \mu\text{mol l}^{-1}$ ) for PI 3-K, and ML-7 ( $100\text{--}1000 \text{ nmol l}^{-1}$ ) for MLCK. These inhibitors did not inhibit PMA-stimulated  $\text{Mg}^{2+}$  efflux (data not shown). Consequently, a role of all these PKs in regulating  $\text{Na}^+/\text{Mg}^{2+}$  antiport is excluded.

From the cumulative findings, it may be allowed to conclude that in non- $\text{Mg}^{2+}$ -loaded rat erythrocytes, PMA can directly stimulate  $\text{Mg}^{2+}$  efflux via  $\text{PKC}_\alpha$  and not via other PKs. It is emerging so far, that in rat erythrocytes the regulation of  $\text{Mg}^{2+}$  efflux via  $\text{Na}^+/\text{Mg}^{2+}$  antiport is different from that in hepatocytes [21,22], MDCT cells [23–26] and other organs where  $\text{Mg}^{2+}$  transport is mainly regulated by PKA.

The regulation of  $\text{Mg}^{2+}$  efflux via  $\text{Na}^+/\text{Mg}^{2+}$  by  $\text{PKC}_\alpha$  differs also from that of  $\text{Na}^+/\text{H}^+$  antiport. In erythrocytes, as in other non-epithelial tissues, only the NHE1 isoform of the  $\text{Na}^+/\text{H}^+$  exchanger (NHE) is expressed [62,63]. In erythrocytes [47], fibroblasts and myocardium, NHE1 has been shown to be activated by the MAPK cascade [64–66].

Since the phosphorylation of the  $\text{Na}^+/\text{Mg}^{2+}$  antiporter should be controlled by the interplay of both  $\text{PKC}_\alpha$  and PPs, we tested whether the inhibition of PPs could enhance the PMA effect. As shown in Table 2, when PKC was already maximally stimulated by PMA, okadaic acid caused a further small increase of  $\text{Mg}^{2+}$  efflux by only 3% in NaCl medium and by 13% in NaSCN medium. Calyculin A caused no further increase of  $\text{Mg}^{2+}$  efflux in both media. Therefore, following maximum stimulation of  $\text{Mg}^{2+}$  efflux by PMA, the additional effect of PPs is small.

### 3.3. Stimulation of $\text{Na}^+$ -independent $\text{Mg}^{2+}$ efflux by PMA in non- $\text{Mg}^{2+}$ -loaded erythrocytes

The effect of PMA on  $\text{Mg}^{2+}$  efflux was also investigated in KCl medium and KSCN medium, wherein  $\text{Na}^+/\text{Mg}^{2+}$  antiport is not active. As in  $\text{Na}^+$ -containing media,  $1 \mu\text{mol l}^{-1}$  PMA also stimulated  $\text{Mg}^{2+}$  efflux in  $\text{K}^+$ -containing media (Fig. 7). This was by 76% in KCl medium and by 62% in KSCN medium. In both  $\text{K}^+$ -containing media,  $\text{Mg}^{2+}$

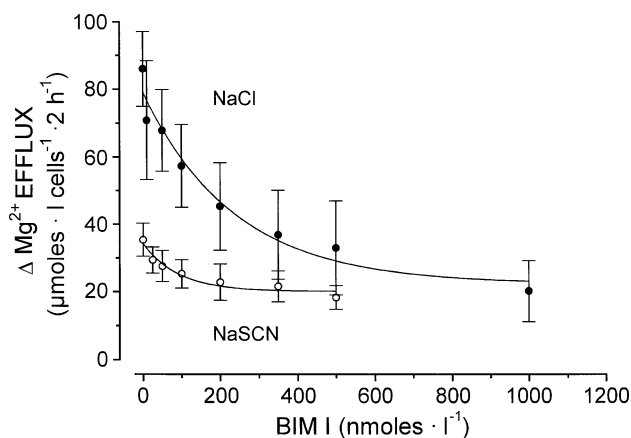


Fig. 6. Inhibition of PMA-stimulated  $\text{Mg}^{2+}$  efflux ( $\Delta\text{Mg}^{2+}$  efflux) of non- $\text{Mg}^{2+}$ -loaded rat erythrocytes suspended in NaCl medium or NaSCN medium by BIM I.  $\Delta\text{Mg}^{2+}$  efflux was calculated as the difference of  $\text{Mg}^{2+}$  efflux at the particular concentration of BIM I plus PMA minus  $\text{Mg}^{2+}$  efflux of the controls without PMA and without BIM I. Means±S.E.,  $n=4\text{--}9$ .

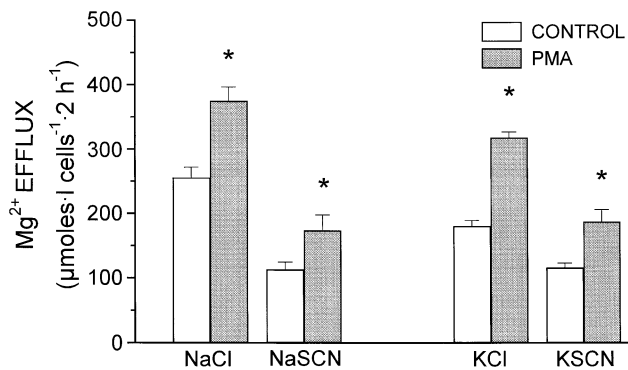


Fig. 7.  $\text{Mg}^{2+}$  efflux of non- $\text{Mg}^{2+}$ -loaded rat erythrocytes suspended in NaCl, NaSCN, KCl and KSCN medium without and with the addition of  $1 \mu\text{mol l}^{-1}$  PMA. Means  $\pm$  S.E.,  $n=4$ . \* $P<0.05$  for PMA vs. control.

efflux might be mediated by  $\text{K}^+/\text{Mg}^{2+}$  exchange via the unspecific choline exchanger [67]. We therefore suggest that in addition to  $\text{Na}^+/\text{Mg}^{2+}$  antiport, the choline exchanger can also be regulated by the interplay of PKs and PPs.

### 3.4. Effect of ATP

In our first experiments on  $\text{Mg}^{2+}$  efflux, we found that  $\text{Mg}^{2+}$  efflux via  $\text{Na}^+/\text{Mg}^{2+}$  antiport from  $\text{Mg}^{2+}$ -loaded erythrocytes requires ATP [9,10]. Other transport systems too, such as  $\text{Na}^+/\text{H}^+$  antiport,  $\text{Na}^+/\text{Ca}^{2+}$  antiport,  $\text{Na}^+/\text{K}^+/\text{Cl}^-$  symport, and  $\text{K}^+/\text{Cl}^-$  symport, were stimulated by cytosolic ATP [14–17,68]. Although these transporters are not primarily active transporters and do not consume ATP, they are inhibited in cells whose ATP content has been reduced.

#### 3.4.1. $\text{Mg}^{2+}$ efflux from ATP-depleted, non- $\text{Mg}^{2+}$ -loaded erythrocytes

As shown in Fig. 8, following ATP depletion,  $\text{Mg}^{2+}$  efflux in NaCl medium was reduced by about 25%. This indicates that  $\text{Na}^+/\text{Mg}^{2+}$  antiport in non- $\text{Mg}^{2+}$ -loaded erythrocytes is also stimulated by ATP. Moreover, ATP depletion caused a 55% lower stimulation of  $\text{Na}^+/\text{Mg}^{2+}$  antiport by PMA and thus by PKC. Erythrocytes express extremely low passive cation permeability [69]. Hereby, an increase of  $[\text{Na}^+]_i$  within the experimental time, which would decrease the inwardly directed  $\text{Na}^+$  gradient as a driving force for  $\text{Na}^+/\text{Mg}^{2+}$  antiport, can be neglected.

Remarkably, in NaSCN medium, ATP depletion increases  $\text{Mg}^{2+}$  efflux, whereas in NaCl medium,  $\text{Mg}^{2+}$  efflux was reduced by ATP depletion (Fig. 8). This effect may be explained by an enhanced efflux of  $\text{Mg}^{2+}$  together with the highly permeating  $\text{SCN}^-$  for charge compensation. Due to the reduced ATP concentration in the PKC reaction, there was only a small effect of PMA on  $\text{Mg}^{2+}$  efflux in ATP-depleted erythrocytes (Fig. 8). An increased efflux of  $\text{Mg}^{2+}$  together with  $\text{SCN}^-$  may mask a small remaining effect of PMA.

### 3.5. Stimulation of $\text{Mg}^{2+}$ efflux by intracellular $\text{Mg}^{2+}$

We previously reported that in rat erythrocytes  $\text{Mg}^{2+}$  loading drastically enhanced  $\text{Mg}^{2+}$  efflux according to a sigmoidal kinetic, having a Hill coefficient of  $n=2.4$  [2,10]. This result led to the conclusion that three  $\text{Mg}^{2+}$  are simultaneously needed for  $\text{Na}^+/\text{Mg}^{2+}$  antiport. One  $\text{Mg}^{2+}$  may be exchanged for two  $\text{Na}^+$  at the transport site, while the other two intracellular  $\text{Mg}^{2+}$  may be bound to a modifier site of the  $\text{Na}^+/\text{Mg}^{2+}$  antiporter, resulting in an allosteric activation of the antiporter [2,10].

This study again revealed a drastic increase of  $\text{Mg}^{2+}$  efflux by  $\text{Mg}^{2+}$  loading (Table 3). As already found, ATP depletion reduced  $\text{Mg}^{2+}$  efflux in  $\text{Mg}^{2+}$ -loaded rat erythrocytes [10]. With ATP-depleted and differently  $\text{Mg}^{2+}$ -loaded rat erythrocytes, we found the same Hill coefficient ( $n=2.4$ ) as in non-ATP-depleted,  $\text{Mg}^{2+}$ -loaded rat erythrocytes. The log  $K$  value of the Hill plot was higher in ATP-depleted cells ( $\Delta\log K=0.6$ , not shown). This corresponds to results obtained with reversed  $\text{Na}^+/\text{Mg}^{2+}$  antiport of ATP-depleted rat erythrocytes [13]. The affinity of the  $\text{Mg}^{2+}$  binding sites on the  $\text{Na}^+/\text{Mg}^{2+}$  antiporter to  $\text{Mg}^{2+}$  is thus dependent on intracellular ATP. A suitable model has already been published [2]. Recently, the same mechanism of ATP depletion was found for the  $\text{Na}^+/\text{H}^+$  antiporter. ATP depletion decreased the  $\text{H}^+$  affinity of the internal modifier site [70].

To determine whether PKs/PPs are additionally involved in the activation of  $\text{Na}^+/\text{Mg}^{2+}$  antiport by intracellular  $\text{Mg}^{2+}$ , we investigated the effect of PMA and staurosporine on  $\text{Mg}^{2+}$  efflux from  $\text{Mg}^{2+}$ -loaded erythrocytes. Fig. 9 shows the time course of  $\text{Mg}^{2+}$  efflux in NaCl and NaSCN medium without and with PMA in erythrocytes loaded in the presence of  $12 \text{ mmol l}^{-1} \text{Mg}^{2+}$ . PMA had no significant effect on  $\text{Mg}^{2+}$  efflux in both media. As can be seen in Fig. 9, in NaSCN medium a constant lower  $\text{Mg}^{2+}$  efflux was obtained as compared with NaCl medium. The failing effect of PMA to significantly stimulate  $\text{Mg}^{2+}$  efflux was not due

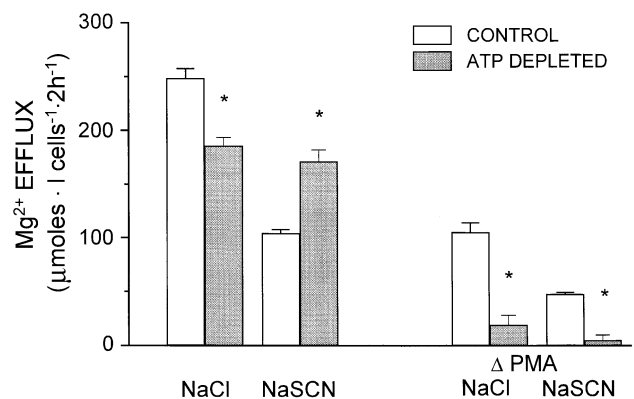


Fig. 8.  $\text{Mg}^{2+}$  efflux of non- $\text{Mg}^{2+}$ -loaded control and ATP-depleted rat erythrocytes suspended in NaCl, NaSCN, KCl or KSCN medium without and in the presence of  $1 \mu\text{mol l}^{-1}$  PMA.  $\Delta\text{PMA}$  is the difference of  $\text{Mg}^{2+}$  efflux in the presence and the absence of PMA. Means  $\pm$  S.E.,  $n=4$ . \* $P<0.05$  for ATP depleted vs. control.

Table 3

Na<sup>+</sup>-dependent and Na<sup>+</sup>-independent Mg<sup>2+</sup> efflux from non-Mg<sup>2+</sup>-loaded and Mg<sup>2+</sup>-loaded rat erythrocytes (RBC)

Group	NaCl	NaSCN	NaCl/NaSCN	KCl	KSCN	KCl/KSCN
Non Mg <sup>2+</sup> -loaded RBC ( $\mu\text{mol l cells}^{-1} 30 \text{ min}^{-1}$ )	44.6 $\pm$ 2.9	19.8 $\pm$ 2.1	2.3	32.2 $\pm$ 0.4	20.3 $\pm$ 1.3	1.6
Mg <sup>2+</sup> -loaded RBC ( $\mu\text{mol l cells}^{-1} 30 \text{ min}^{-1}$ )	5979 $\pm$ 365	5063 $\pm$ 311	1.2	299 $\pm$ 8.4	384 $\pm$ 8.8	0.8
Mg <sup>2+</sup> -loaded RBC/Non Mg <sup>2+</sup> -loaded RBC	134	256		9.3	18.9	

The RBC were suspended in NaCl, NaSCN, KCl, and KSCN medium. Mg<sup>2+</sup> loading of RBC was done in the presence of 12 mmol l<sup>-1</sup> MgCl<sub>2</sub>. Means  $\pm$  S.E. of four experiments.

to the experimental procedure of Mg<sup>2+</sup> loading, since in non-Mg<sup>2+</sup>-loaded erythrocytes, treatment with A 23187 or with the KCl albumin-containing washing solution did not offset the PMA stimulation of Mg<sup>2+</sup> efflux (data not shown).

The absence of a stimulatory effect of PMA on Mg<sup>2+</sup> efflux in Mg<sup>2+</sup>-loaded erythrocytes may have two reasons: First, since the high [Mg<sup>2+</sup>]<sub>i</sub> may compete with [Ca<sup>2+</sup>]<sub>i</sub>, it may thereby prevent the activation of the Ca<sup>2+</sup>-dependent PKC $\alpha$ . Second, Na<sup>+</sup>/Mg<sup>2+</sup> antiport may be already fully activated by Mg<sup>2+</sup> loading, whereby a possible additive effect of PMA is masked.

In order to test whether Mg<sup>2+</sup> loading itself activates PKs, the effect of several PK inhibitors was tested in Mg<sup>2+</sup>-loaded erythrocytes. Staurosporine (1  $\mu\text{mol l}^{-1}$ ), BIM I (0.5–1.0  $\mu\text{mol l}^{-1}$ ) and Ro-31-8425 (0.1–1.0  $\mu\text{mol l}^{-1}$ ) had no significant effect on Mg<sup>2+</sup> efflux (data not shown). Therefore, in the drastic activation of Mg<sup>2+</sup> efflux by Mg<sup>2+</sup> loading, an activation of PKC $\alpha$  and other PKs is not involved. Also, inhibition of PPs by 1  $\mu\text{mol l}^{-1}$  calyculin A had no effect on Mg<sup>2+</sup> efflux of Mg<sup>2+</sup>-loaded rat erythrocytes (not shown).

Based on these results, we conclude that the activation of Mg<sup>2+</sup> efflux by Mg<sup>2+</sup> loading is caused by a direct effect of intracellular Mg<sup>2+</sup> together with ATP either on the Na<sup>+</sup>/Mg<sup>2+</sup> antiporter or an associated regulatory protein.

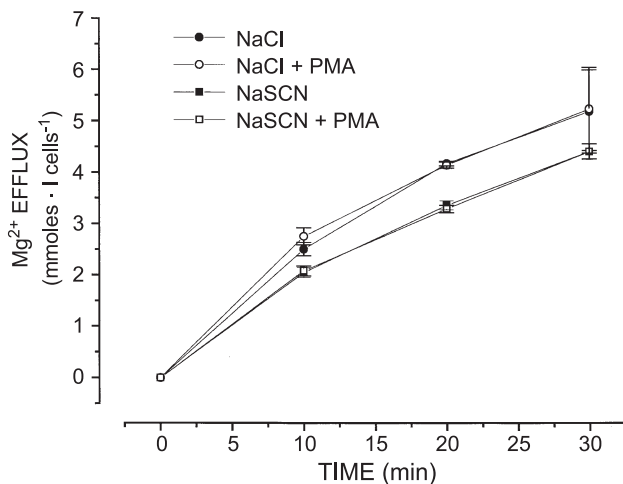


Fig. 9. Time dependence of Mg<sup>2+</sup> efflux of rat erythrocytes loaded with 12 mmol l<sup>-1</sup> Mg<sup>2+</sup> in the Mg<sup>2+</sup> loading medium. Mg<sup>2+</sup> efflux was measured in NaCl medium and NaSCN medium without or in the presence of 1  $\mu\text{mol l}^{-1}$  PMA. Means  $\pm$  S.E.,  $n = 3-5$ .

### 3.5.1. Na<sup>+</sup>-independent Mg<sup>2+</sup> efflux in Mg<sup>2+</sup>-loaded rat erythrocytes

Mg<sup>2+</sup> loading increased Na<sup>+</sup>-independent Mg<sup>2+</sup> efflux (Mg<sup>2+</sup> efflux in KCl and KSCN medium) in parallel to the increased Mg<sup>2+</sup> gradient (about 9 to 19 times, respectively). Na<sup>+</sup>-dependent Mg<sup>2+</sup> efflux through Na<sup>+</sup>/Mg<sup>2+</sup> antiport was increased by a factor of 134 and 256 in NaCl and NaSCN medium, respectively (Table 3).

### 3.6. Role of intracellular Cl<sup>-</sup> in the regulation of Mg<sup>2+</sup> efflux

We previously showed that in non-Mg<sup>2+</sup>-loaded erythrocytes suspended in NaSCN medium with zero [Cl<sup>-</sup>]<sub>i</sub>, there was a residual Mg<sup>2+</sup> efflux amounting to 30–40% of Mg<sup>2+</sup> efflux in NaCl medium [32]. With increasing [Cl<sup>-</sup>]<sub>i</sub>, produced by the substitution of NaSCN by NaCl, Mg<sup>2+</sup> efflux was stimulated according to a sigmoidal kinetic having a Hill coefficient for [Cl<sup>-</sup>]<sub>i</sub> of 1.6–1.8 [32]. The Mg<sup>2+</sup> efflux remaining at zero [Cl<sup>-</sup>]<sub>i</sub> may be caused by [Cl<sup>-</sup>]<sub>i</sub>-independent Na<sup>+</sup>/Mg<sup>2+</sup> antiport or by Mg<sup>2+</sup> efflux through another pathway, e.g. through the unspecific choline antiporter, which also accepts other cations instead of choline [67]. Calculating the difference of Mg<sup>2+</sup> efflux in NaCl medium minus KCl medium from Fig. 7 yielded 75.4  $\mu\text{mol l cells}^{-1} 2 \text{ h}^{-1}$ . The difference of Mg<sup>2+</sup> efflux in NaSCN medium minus KSCN medium amounted to  $-2.4 \mu\text{mol l cells}^{-1} 2 \text{ h}^{-1}$ . This result shows that Na<sup>+</sup>/Mg<sup>2+</sup> antiport in non-Mg<sup>2+</sup>-loaded erythrocytes essentially depends on intracellular Cl<sup>-</sup>. Mg<sup>2+</sup> efflux in NaSCN, KCl, and KSCN medium can be explained by Na<sup>+</sup>/Mg<sup>2+</sup> exchange and K<sup>+</sup>/Mg<sup>2+</sup> exchange through the unspecific choline exchanger [67]. Moreover, Fig. 7 shows that PMA stimulated Mg<sup>2+</sup> efflux in the absence of intracellular Cl<sup>-</sup> in NaSCN medium and KSCN medium. From these findings, we conclude that the stimulation of Na<sup>+</sup>-dependent and Na<sup>+</sup>-independent Mg<sup>2+</sup> efflux by PMA does not depend on [Cl<sup>-</sup>]<sub>i</sub>.

The role of [Cl<sup>-</sup>]<sub>i</sub> in the activation of Mg<sup>2+</sup> efflux by PMA at elevated [Mg<sup>2+</sup>]<sub>i</sub> was also investigated in Mg<sup>2+</sup>-loaded erythrocytes suspended in NaSCN medium. Fig. 9 shows that PMA had no significant effect on Mg<sup>2+</sup> efflux in NaSCN medium, as was found for NaCl medium. As with non-Mg<sup>2+</sup>-loaded erythrocytes, Mg<sup>2+</sup> efflux in NaSCN medium was lower than in NaCl medium. However, in Mg<sup>2+</sup>-loaded erythrocytes the difference and ratio between Mg<sup>2+</sup> efflux in NaCl and NaSCN medium was smaller than that in non-Mg<sup>2+</sup>-loaded erythrocytes (Fig. 9, Table 3). It



may be concluded that  $\text{Na}^+/\text{Mg}^{2+}$  antiport in  $\text{Mg}^{2+}$ -loaded erythrocytes may be less dependent on intracellular  $\text{Cl}^-$ . Similarly,  $\text{Mg}^{2+}$  efflux in  $\text{Mg}^{2+}$ -loaded erythrocytes in  $\text{K}^+$ -containing medium, which may operate through the choline exchanger by  $\text{K}^+/\text{Mg}^{2+}$  exchange, was also less dependent on intracellular  $\text{Cl}^-$  (Table 3). These effects may be explained by the different lyotropic properties of  $\text{SCN}^-$  and  $\text{Cl}^-$  according to the Hofmeister series, although the exact mechanism of interaction of  $\text{Mg}^{2+}$  and both anions with the antiporters is not known.

In conclusion, the drastic activation of  $\text{Na}^+/\text{Mg}^{2+}$  antiport by intracellular  $\text{Mg}^{2+}$  overwhelms the other activating regulatory mechanisms of activation by  $[\text{Cl}^-]_i$  and by  $\text{PKC}_\alpha$ /protein phosphatases because the  $\text{Na}^+/\text{Mg}^{2+}$  antiport in  $\text{Mg}^{2+}$ -loaded erythrocytes is operating at its maximal capacity.

### 3.7. Exclusion of TRPM7 as $\text{Na}^+/\text{Mg}^{2+}$ antiporter

As shown by kinetic analysis,  $\text{Na}^+/\text{Mg}^{2+}$  antiport must be mediated by a single protein and not by the combination of a  $\text{Na}^+$ -transporting and a  $\text{Mg}^{2+}$ -transporting protein [9,71]. Its structure is yet to be characterized. It may be asked whether the newly cloned and functionally characterized TRPM7 performs  $\text{Na}^+/\text{Mg}^{2+}$  antiport. The main differences between TRPM7 and the  $\text{Na}^+/\text{Mg}^{2+}$  antiporter are as follows.

TRPM7 is a bifunctional protein with intrinsic cation channel and PK domains. It acts as a nonspecific metal ion pathway, conducting divalent cations into the cell. At positive potentials and submillimolar  $\text{Mg}^{2+}$  concentrations, it also mediates influx or efflux of monovalent cations [72–75]. The  $\text{Na}^+/\text{Mg}^{2+}$  antiporter is an electroneutral antiporter that under physiological conditions exchanges extracellular  $\text{Na}^+$  for intracellular  $\text{Mg}^{2+}$ .

$\text{Mg}^{2+}$  influx via TRPM7 is inhibited by intracellular Mg and Mg-ATP [72]. As shown previously [9,13] and in this study,  $\text{Na}^+/\text{Mg}^{2+}$  antiport is drastically activated by intracellular Mg and ATP.

The  $\alpha$ -kinase associated with TRPM7 is not sensitive to staurosporine [76], but is inhibited by H89 [77]. As shown in this study, the opposite is true for  $\text{Na}^+/\text{Mg}^{2+}$  antiport. This excludes that the unidentified PK affecting  $\text{Na}^+/\text{Mg}^{2+}$  antiport is identical to the  $\alpha$ -kinase of TRPM7.

From the cumulative evidence, we conclude that TRPM7 is not identical to  $\text{Na}^+/\text{Mg}^{2+}$  antiporter.

### 3.8. Functional consequences

$\text{Mg}^{2+}$  efflux is low in untreated erythrocytes. This low  $\text{Mg}^{2+}$  efflux is regulated by  $[\text{Cl}^-]_i$ , ATP, and by the interplay of unidentified PK activity with PPs. A rather constant  $[\text{Mg}^{2+}]_i$  can thereby be maintained. The most drastic regulation of  $\text{Mg}^{2+}$  efflux is induced by intracellular  $\text{Mg}^{2+}$ . When  $[\text{Mg}^{2+}]_i$  is increased, e.g. by  $\text{Mg}^{2+}$  loading or in hypermagnesemia and, in case of ATP depletion as in hypoxia and acidosis, a drastic increase of  $\text{Mg}^{2+}$  efflux

through  $\text{Na}^+/\text{Mg}^{2+}$  antiport is induced. For details of the mechanism, see Ref. [2]. Thus, the physiological concentration of  $[\text{Mg}^{2+}]_i$  will be rapidly reached. An optimal function of the  $\text{Mg}^{2+}$ -dependent enzymes is thereby established. Furthermore, in nucleated cells, miscoding is prevented and the action of initiation and dissociation factors in protein biosynthesis is facilitated [78].

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### References

- [1] H. Ebel, T. Günther, Characterization of  $\text{Mg}^{2+}$  efflux from rat erythrocytes non-loaded with  $\text{Mg}^{2+}$ , *Biochim. Biophys. Acta* 1421 (1999) 353–360.
- [2] T. Günther, Putative mechanism of  $\text{Mg}^{2+}/\text{Mg}^{2+}$  exchange and  $\text{Na}^+/\text{Mg}^{2+}$  antiport, *Magnesium-Bull.* 18 (1996) 2–6.
- [3] L. Bianchini, M. Woodside, C. Sardet, J. Pouyssegur, A. Takai, S. Grinstein, Okadaic acid, a phosphatase inhibitor, induces activation and phosphorylation of the  $\text{Na}^+/\text{H}^+$  antiport, *J. Biol. Chem.* 266 (1991) 15406–15413.
- [4] C. Sardet, L. Counillon, A. Franchi, J. Pouyssegur, Growth factors induce phosphorylation of the  $\text{Na}^+/\text{H}^+$  antiporter, glycoprotein of 110 kD, *Science* 247 (1990) 723–726.
- [5] S.A. Levine, M.H. Montrose, C.M. Tse, M. Donowitz, Kinetics and regulation of three cloned mammalian  $\text{Na}^+/\text{H}^+$  exchangers stably expressed in a fibroblast cell line, *J. Biol. Chem.* 268 (1993) 25527–25535.
- [6] J.P. Reeves, Molecular aspects of sodium–calcium exchange, *Arch. Biochem. Biophys.* 292 (1992) 329–334.
- [7] P. Caroni, E. Carafoli, The regulation of the  $\text{Na}^+/\text{Ca}^{2+}$  exchanger of heart sarcolemma, *Eur. J. Biochem.* 132 (1983) 451–460.
- [8] R. DiPolo, L. Beaugé, In squid axons, ATP modulates  $\text{Na}^+/\text{Ca}^{2+}$  exchange by a  $\text{Ca}^{2+}$  dependent phosphorylation, *Biochim. Biophys. Acta* 897 (1987) 347–354.
- [9] T. Günther, J. Vormann, R. Förster, Regulation of intracellular magnesium by  $\text{Mg}^{2+}$  efflux, *Biochem. Biophys. Res. Commun.* 119 (1984) 124–131.
- [10] T. Günther, J. Vormann, V. Höllriegel, Characterization of  $\text{Na}^+$ -dependent  $\text{Mg}^{2+}$  efflux from  $\text{Mg}^{2+}$ -loaded rat erythrocytes, *Biochim. Biophys. Acta* 1023 (1990) 455–461.
- [11] J.C. Feray, R. Garay, An  $\text{Na}^+$ -stimulated  $\text{Mg}^{2+}$ -transport system in human red blood cells, *Biochim. Biophys. Acta* 856 (1986) 76–84.
- [12] E.J. Frenkel, M. Graziani, H.J. Schatzmann, ATP requirement of the sodium-dependent magnesium extrusion from human red blood cells, *J. Physiol.* 414 (1989) 385–397.
- [13] T. Günther, J. Vormann, Reversibility of  $\text{Na}^+/\text{Mg}^{2+}$  antiport in rat erythrocytes, *Biochim. Biophys. Acta* 1234 (1995) 105–110.
- [14] D.W. Hilgemann, Cytoplasmic ATP-dependent regulation of ion transporters and channels: mechanisms and messengers, *Annu. Rev. Physiol.* 59 (1997) 193–220.
- [15] P.W. Flatman, Regulation of Na-K-2Cl cotransport by phosphorylation and protein–protein interactions, *Biochim. Biophys. Acta* 1566 (2002) 140–151.
- [16] D.H. Schulze, M. Muqhal, W.J. Lederer, A.M. Ruknudin, Sodium/calcium exchanger (NCX1) macromolecular complex, *J. Biol. Chem.* 278 (2003) 28849–28855.
- [17] B.F. Dowd, B. Forbush, PASK (proline-alanine-rich STE20-related

- kinase), a regulatory kinase of the Na-K-Cl cotransporter (NKCC1), *J. Biol. Chem.* 278 (2003) 27347–27353.
- [18] T. Günther, J. Vormann, Probable role of protein phosphorylation in the regulation of  $Mg^{2+}$  efflux via  $Na^+/Mg^{2+}$  antiport, *Magnesium-Bull.* 8 (1986) 307–309.
  - [19] T. Günther, J. Vormann, Activation of  $Na^+/Mg^{2+}$  antiport in thymocytes by cAMP, *FEBS Lett.* 297 (1992) 132–134.
  - [20] F.I. Wolf, A. Di Francesco, V. Covacci, A. Cittadini, cAMP activates magnesium efflux via the Na/Mg antiporter in ascites cells, *Biochem. Biophys. Res. Commun.* 202 (1994) 1209–1214.
  - [21] A. Romani, A. Scarpa, Norepinephrine evokes a marked  $Mg^{2+}$  efflux from liver cells, *FEBS Lett.* 269 (1990) 37–40.
  - [22] T. Günther, J. Vormann, V. Höllriegel, Noradrenaline-induced  $Na^+$ -dependent  $Mg^{2+}$  efflux from rat liver, *Magnesium-Bull.* 13 (1991) 122–124.
  - [23] H.S. Kang, D. Kerstan, L.J. Dai, G. Ritchie, G.A. Quamme, beta-Adrenergic agonists stimulate  $Mg^{2+}$  uptake in mouse distal convoluted tubule cells, *Am. J. Physiol.* 279 (2000) F1116–F1123.
  - [24] L.J. Dai, G.A. Quamme, Cyclic nucleotides alter intracellular free  $Mg^{2+}$  in renal epithelial cells, *Am. J. Physiol.* 262 (1992) F1100–F1104.
  - [25] L.J. Dai, B. Bapty, G. Ritchie, G.A. Quamme,  $PGE_2$  stimulates  $Mg^{2+}$  uptake in mouse distal convoluted tubule cells, *Am. J. Physiol.* 275 (1998) F833–F839.
  - [26] H.S. Kang, D. Kerstan, L.J. Dai, G. Ritchie, G.A. Quamme, Adenosine modulates  $Mg^{2+}$  uptake in distal convoluted tubule cells via A(1) and A(2) purinoceptors, *Am. J. Physiol.* 281 (2001) F1141–F1147.
  - [27] J. Vormann, T. Günther, Amiloride-sensitive net  $Mg^{2+}$  efflux from isolated perfused rat hearts, *Magnesium* 6 (1987) 220–224.
  - [28] A. Romani, A. Scarpa, Hormonal control of  $Mg^{2+}$  transport in the heart, *Nature* 346 (1990) 841–844.
  - [29] A. Jakob, J. Becker, G. Schöttli, G. Fritzsche,  $\alpha_1$ -Adrenergic stimulation causes  $Mg^{2+}$  release from perfused rat liver, *FEBS Lett.* 246 (1989) 127–130.
  - [30] T.E. Fagan, A. Romani,  $\alpha_1$ -Adrenoceptor-induced  $Mg^{2+}$  extrusion from rat hepatocytes occurs via  $Na^+$ -dependent transport mechanism, *Am. J. Physiol.* 280 (2001) G1145–G1156.
  - [31] A. Ikari, K. Nakajima, S. Taki, Y. Suketa, Up-regulation of  $Na^+$ -dependent  $Mg^{2+}$  transport by nitric oxide and cyclic GMP pathway in renal epithelial cells, *Eur. J. Pharmacol.* 451 (2002) 133–139.
  - [32] H. Ebel, T. Günther, Stimulation of  $Na^+/Mg^{2+}$  antiport in rat erythrocytes by intracellular  $Cl^-$ , *FEBS Lett.* 543 (2003) 103–107.
  - [33] C. Lytle, T. McManus, Coordinate modulation of Na-K-2Cl cotransport and K-Cl cotransport by cell volume and chloride, *Am. J. Physiol.* 283 (2002) C1422–C1431.
  - [34] G. Fairbanks, J. Avruch, Phosphorylation of endogenous substrates by erythrocyte membrane protein kinases: II. Cyclic adenosine monophosphate-stimulated reactions, *Biochemistry* 13 (1974) 5514–5521.
  - [35] M.M. Hosey, M. Tao, An analysis of the autophosphorylation of rabbit and human erythrocyte membranes, *Biochemistry* 15 (1976) 1561–1568.
  - [36] H.K. Jindal, Z. Ai, P. Gascard, C. Horten, C.M. Cohen, Specific loss of protein kinase activities in senescent erythrocytes, *Blood* 88 (1996) 1479–1486.
  - [37] R.S. Sprague, M.L. Ellsworth, A.H. Stephenson, A.J. Lonigro, Participation of cAMP in a signal-transduction pathway relating erythrocyte deformation to ATP release, *Am. J. Physiol.* 281 (2001) C1158–C1164.
  - [38] K. Quiring, G. Kaiser, D. Gauger, D. Palm, Cyclic AMP-dependent protein kinases and binding sites for cyclic AMP in rat erythrocytes, *Naunyn-Schmiedeberg's Arch. Pharmacol.* 290 (1975) 397–417.
  - [39] H. Fatallah, M. Sauvage, J.R. Romero, M. Canessa, F. Giraud, Effects of PKC $\alpha$  activation on  $Ca^{2+}$  pump and K(Ca)channel in deoxygenated sickle cells, *Am. J. Physiol.* 273 (1997) C1206–C1214.
  - [40] R.B. Govekar, S.M. Zingde, Protein kinase C isoforms in human erythrocytes, *Ann. Hematol.* 80 (2001) 531–534.
  - [41] M. Sauvage, P. Mazière, H. Fatallah, F. Giraud, Insulin stimulates NHE1 activity by sequential activation of phosphatidylinositol 3-kinase and protein kinase C $\xi$  in human erythrocytes, *Eur. J. Biochem.* 267 (2000) 955–962.
  - [42] C.C. Wang, M. Tao, T. Wei, P.S. Low, Identification of the major protein kinase I phosphorylation sites on erythrocyte band 3, *Blood* 89 (1997) 3019–3024.
  - [43] T. Wei, M. Tao, Human erythrocyte casein kinase II: characterization and phosphorylation of membrane cytoskeletal proteins, *Arch. Biochem. Biophys.* 307 (1993) 206–216.
  - [44] M.L. Harrison, C.C. Isaacson, D.L. Burg, R.L. Geahlen, P.S. Low, Phosphorylation of human erythrocyte band 3 by endogenous p72syk, *J. Biol. Chem.* 269 (1994) 955–959.
  - [45] A.M. Brunati, L. Bordin, G. Clari, V. Moret, The Lyn-catalysed Tyr phosphorylation of the transmembrane band-3 protein of human erythrocytes, *Eur. J. Biochem.* 240 (1996) 394–399.
  - [46] S.J. Kelley, R. Thomas, P.B. Dunham, Candidate inhibitor of the volume-sensitive kinase regulating K-Cl cotransport: the myosin light chain kinase inhibitor ML-7, *J. Membr. Biol.* 178 (2000) 31–41.
  - [47] M. Sartori, G. Ceolotto, A. Semplicini, MAPKinase and regulation of sodium-proton exchanger in human red blood cells, *Biochim. Biophys. Acta* 1421 (1999) 140–148.
  - [48] Y. Zipser, N.S. Kosower, Phosphotyrosine phosphatase associated with band 3 protein in the human erythrocyte membrane, *Biochem. J.* 314 (1996) 881–887.
  - [49] L. De Franceschi, E. Villa-Moruzzi, L. Fumagalli, C. Brugnara, F. Turrini, R. Motta, E. Veghini, C. Corato, S.L. Alper, G. Berton, K-Cl cotransport modulation by intracellular Mg in erythrocytes from mice bred for low and high Mg levels, *Am. J. Physiol.* 281 (2001) C1385–C1395.
  - [50] I. Bize, B. Guvenc, A. Robb, G. Buchbinder, C. Brugnara, Serine/threonine protein phosphatases and regulation of K-Cl cotransport in human erythrocytes, *Am. J. Physiol.* 277 (1999) C926–C936.
  - [51] A.N. Nambodiripad, M.L. Jennings, Permeability characteristics of erythrocyte membrane to okadaic acid and calyculin A, *Am. J. Physiol.* 270 (1996) C449–C456.
  - [52] Y. Zipser, A. Piade, A. Barbul, R. Korenstein, N.S. Kosower,  $Ca^{2+}$  promotes erythrocyte band 3 tyrosine phosphorylation via dissociation of phosphotyrosine phosphatase from band 3, *Biochem. J.* 368 (2002) 137–144.
  - [53] D.C. Crans, Vanadate–protein interactions, in: G. Berthon (Ed.), *Handbook of Metal–Ligand Interactions in Biological Fluids*, Bioinorganic Chemistry, vol. I, Marcel Dekker, New York, 1995, pp. 267–273.
  - [54] I.G. Macara, K. Kustin, L.C. Cantley Jr., Glutathione reduces cytoplasmic vanadate. Mechanism and physiological implications, *Biochim. Biophys. Acta* 629 (1980) 95–106.
  - [55] D.C. Crans, C.M. Simone, A.K. Saha, R.H. Glew, Vanadate monomers and dimers both inhibit the human prostatic acid phosphatase, *Biochem. Biophys. Res. Commun.* 165 (1989) 246–250.
  - [56] V. López, T. Stevens, R.N. Lindquist, Vanadium ion inhibition of alkaline phosphatase-catalysed phosphate ester hydrolysis, *Arch. Biochem. Biophys.* 175 (1976) 31–38.
  - [57] P.J. Stankiewicz, M.J. Gresser, Inhibition of phosphatase and sulfatase by transition-state analogues, *Biochemistry* 27 (1988) 206–212.
  - [58] J.E. Benabe, L.A. Echegoyen, B. Pastrana, M. Martinez-Maldonado, Mechanism of inhibition of glycolysis by vanadate, *J. Biol. Chem.* 262 (1987) 9555–9560.
  - [59] M. Barbagallo, L.J. Dominguez, L.M. Resnick, Insulin-mimetic action of vanadate: role of intracellular magnesium, *Hypertension* 38 (2001) 701–704.
  - [60] P. Lijnen, D. Echevaria-Vázquez, R. Fagard, V. Petrov, Protein kinase C induced changes in erythrocyte  $Na^+/H^+$  exchange and cytosolic free calcium in humans, *Am. J. Hypertens.* 11 (1998) 81–87.
  - [61] D. Ron, M.G. Kazanietz, New insights into the regulation of protein kinase C and novel phorbol ester receptors, *FASEB J.* 13 (1999) 1658–1676.
  - [62] P.A. Rutherford, J.H. Pizzonia, D. Biemesderfer, A. Abu-Alfa, R. Reilly, P.S. Aronson, Expression of  $Na^+-H^+$  exchanger isoforms

- NHE1 and NHE3 in kidney and blood cells of rabbit and rat, *Exp. Nephrol.* 5 (1997) 490–497.
- [63] R. Sarangarajan, N. Dhabia, M. Soliemani, N. Baird, C. Joiner, NHE-1 is the sodium–hydrogen exchanger isoform present in erythroid cells, *Biochim. Biophys. Acta* 1374 (1998) 56–62.
- [64] L. Bianchini, G. L'Allemain, J. Pousségur, The p42/p44 mitogen-activated protein kinase cascade is determinant in mediating activation of the  $\text{Na}^+/\text{H}^+$  exchanger (NHE1 isoform) in response to growth factors, *J. Biol. Chem.* 272 (1997) 271–279.
- [65] A.N. Moor, L. Fliegel, Protein kinase-mediated regulation of the  $\text{Na}^+/\text{H}^+$  exchanger in the rat myocardium by mitogen-activated protein kinase-dependent pathways, *J. Biol. Chem.* 274 (1999) 22985–22992.
- [66] O. Aharonovitz, Y. Granot, Stimulation of mitogen-activated protein kinase and  $\text{Na}^+/\text{H}^+$  exchanger in human platelets. Differential effect of phorbol ester and vasopressin, *J. Biol. Chem.* 271 (1996) 16494–16499.
- [67] H. Ebel, M. Hollstein, T. Günther, Role of the choline exchanger in  $\text{Na}^+$ -independent  $\text{Mg}^{2+}$  efflux from rat erythrocytes, *Biochim. Biophys. Acta* 1559 (2002) 135–144.
- [68] J.R. Sachs, D.W. Martin, The role of ATP in swelling-stimulated K-Cl cotransport in human red cell ghosts. Phosphorylation–dephosphorylation events are not in the signal transduction pathway, *J. Gen. Physiol.* 102 (1993) 551–573.
- [69] J.E. Harris, The influence of the metabolism of human erythrocytes on their potassium content, *J. Biol. Chem.* 141 (1941) 579–595.
- [70] S. Wakabayashi, T. Hisamitsu, T. Pang, M. Shigekawa, Kinetic dissection of two distinct proton binding sites in  $\text{Na}^+/\text{H}^+$  exchangers by measurement of reverse mode reaction, *J. Biol. Chem.* 278 (2003) 43580–43585.
- [71] T. Günther, J. Vormann,  $\text{Mg}^{2+}$  efflux is accomplished by an amiloride-sensitive  $\text{Na}^+/\text{Mg}^{2+}$  antiport, *Biochem. Biophys. Res. Commun.* 130 (1985) 540–545.
- [72] M.J.S. Nadler, M.C. Hermosura, K. Inabe, A.L. Perraud, Q. Zhu, A.J. Stokes, T. Kurosaki, J.P. Kinet, R. Penner, A.M. Scharenberg, A. Fleig, LTRPC7 is a Mg ATP-regulated divalent cation channel required for cell viability, *Nature* 411 (2001) 590–595.
- [73] L.W. Runnels, L. Yue, D.E. Clapham, TRP-PLIK, a bifunctional protein with kinase and ion channel activities, *Science* 291 (2001) 1043–1047.
- [74] M.K. Monteilh-Zoller, M.C. Hermosura, M.J.S. Nadler, A.M. Scharenberg, R. Penner, A. Fleig, TRPM provides an ion channel for cellular entry of trace metal ions, *J. Gen. Physiol.* 121 (2002) 49–60.
- [75] C. Schmitz, A.L. Perraud, C.O. Johnson, K. Inabe, M.K. Smith, R. Penner, T. Kurosaki, A. Fleig, A.M. Scharenberg, Regulation of vertebrate cellular  $\text{Mg}^{2+}$  homeostasis by TRPM7, *Cell* 114 (2003) 191–200.
- [76] L.V. Ryazanova, M.V. Dorovkov, A. Ansari, A.G. Ryazanov, Characterization of the protein kinase activity of TRPM7/ChaK1, a protein kinase fused to the transient receptor potential ion channel, *J. Biol. Chem.* 279 (2004) 3708–3716.
- [77] R. Takezawa, C. Schmitz, P. Demeuse, A.M. Scharenberg, R. Penner, A. Fleig, Receptor-mediated regulation of the TRPM7 channel through its endogenous protein kinase domain, *Proc. Natl. Acad. Sci. U.S.A.* 101 (2004) 6009–6014. Epub 2004, Apr. 06.
- [78] H. Ebel, T. Günther, Magnesium metabolism. A review, *J. Clin. Chem. Clin. Biochem.* 18 (1980) 257–270.